A Comparison of Phosphate Buffered and Distilled Water Dilution Blanks for the Standard Plate Count of Raw-Milk Bacteria[1]

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ABSTRACT

Raw milk samples were diluted with distilled water or distilled water with added phosphate buffer as recommended by *Standard Methods for the Examination of Dairy Products*. The standard plate counts were higher in diluent without phosphate buffer with both high and low count milk. The higher counts were significant when analyzed by a nonparametric sign test or a t-test of differences but were not significant with an analysis of variance technique. Reproducibility was not statistically different in the two diluents. It is suggested that the use of phosphate buffer for raw milk bacteria counts be discontinued until information showing definite advantages is provided.

Use of dilution water fortified with buffers was originally developed for studying biochemical oxygen demand in polluted waters that might not support adequate bacterial growth due to lack of necessary minerals or to high pH. Mohlman et al. (*7*) proposed a dilution water containing 500 ppm $NaHCO_3$ (bicarbonate water). Theriault et al. (*9*) compared distilled water, bicarbonate water, and phosphate buffer [1.25 ml per liter of stock solution of 34.0 g KH_2PO_4 in one liter of distilled water with the pH adjusted to 7.2 with 1 N NaOH—the same concentration now recommended by *Standard Methods* (*1*)]. These authors (*9*) concluded that phosphate buffer by itself was a suitable diluent for biochemical oxygen demand analyses of polluted waters.

Butterfield (*3*) extended the buffered dilution blank studies to the isolation of bacteria from the water of seven rivers or creeks. He found a high pH (7.6-8.2) in six of the waters after autoclaving and these remained high for 48 h. Distilled water also became alkaline but reverted to pH 7.2 in 48 h. Phosphate buffered distilled water with or without the added minerals remained at a constant pH (7.4). Bacterial counts were obtained in an unspecified agar incubated at 37 C. Highest counts were obtained using the above fortified phosphate buffer although phosphate buffer alone was nearly as good. Lowest counts were obtained with bicarbonate water with distilled water being somewhat better but not as good as phosphate alone.

As far as we know, the practice of using phosphate buffer for isolating bacteria from dairy products stems from the studies of the above workers on water-borne bacteria. The only reference to its use in *Standard Methods* is the paper by Butterfield (*3*). We undertook the study reported here because of the lack of data on the efficacy of phosphate buffer as a diluent for bacteria in dairy products.

MATERIALS AND METHODS

Methods advocated by *Standard Methods* (*1*) were followed with the exception of the distilled water [nonphosphate buffered (NPB)] series. This was a collaborative assay by the members of the Subcommittee for the Examination of Milk and Milk Products, Applied Laboratory Methods Committee, International Association of Milk, Food, and Environmental Sanitarians, Inc. Nine analysts secured their own raw milk samples (total of 82 for this study) which were from the states of Minnesota, Louisiana, Tennessee, North Carolina, Ohio, and Texas. Distilled water (NPB) with or without phosphate buffer (PB) at the concentration recommended by *Standard Methods* (*1*) and Theriault et al. (*9*) was used as the diluent. Duplicate petri-dishes were poured and in most instances replicate aliquots of raw milk were plated.

RESULTS

pH measurements

Distilled water pH values reported by the different laboratories ranged from 5.50 to 8.70 with 13 of 19 samples in the range 5.80 to 6.80. Freshly distilled water gave the highest values of 8.55 and 8.70; these, which were used in two days and one day, respectively, showed a drop in pH to 7.35 and 7.50, respectively. The other pH measurements reported were those of distilled water just before addition of milk or buffer. When buffer was added, the pH values reported were 7.05 in 14 cases;

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TABLE 1. *Effect of phosphate buffer in dilution water on plate counts of raw milk bacteria*

Investigator	Milk sample no.	Phosphate (PB) Rep. 1		Rep. 2		No phosphate (NPB) Rep. 1		Rep. 2		Increase (+) or decrease (—) without phosphate[c]
A	1[a]	119[b]	124	146	158	179	168	198	194	+
	2	30.0	27.9	30.9	29.3	27.7	28.2	30.1	27.3	—
	3[a]	398	422	369	389	368	397	386	390	—
	4[a]	205	239	241	234	205	237	240	239	+
	5[a]	113	118	106	110	121	99	103	146	+
	6[a]	71	104	93	104	84	124	93	98	+
	7	13.1	8.4	12.5	11.7	10.7	12.2	14.7	14.6	+
	8[a]	350	319	337	291	314	277	298	267	—
	9	8.8	9.5	9.1	9.1	8.7	7.3	8.4	7.5	—
	10[a]	43	43	56	59	53	41	43	40	—
Arith. mean			139.03				139.98			+
Geom. mean[d]			4.326				4.337			+
B	11	4.2	4.8	4.2	4.5	4.1	5.0	4.0	3.0	—
	12	4.0	3.4	3.6	4.3	3.8	4.4	3.0	2.9	—
	13	6.8	7.6	7.4	8.0	4.7	4.4	6.7	8.1	—
	14[a]	62	57	55	61	62	61	54	40	—
	15	27	30	30	28	28	27	29	30	—
	16	3.9	2.8	3.6	4.2	3.2	3.7	2.0	3.1	—
	17[a]	69	77	72	64	69	57	70	71	—
	18[a]	60	53	50	51	67	72	75	79	+
Arith. mean			28.85				29.91			+
Geom. mean			2.720				2.658			—
C	19	21.0	20.6	21.8	22.9	21.6	19.5	24.4	23.0	+
	20	11.9	10.8	12.5	12.3	12.7	12.6	12.8	13.7	+
	21	15.2	13.8	15.9	17.7	16.3	17.2	17.4	17.9	+
	22	18.5	17.4	20.9	17.2	19.7	20.6	18.7	21.8	+
	23	10.1	9.6	10.3	9.7	8.7	11.2	11.1	9.9	+
	24	8.4	8.6	8.7	7.9	8.9	9.2	8.2	9.1	+
Arith. mean			14.32				15.26			+
Geom. mean			2.604				2.666			+
D	25	14.4	13.6			14.4	14.7			+
	26	6.2	7.2			7.3	7.4			+
	27	14.1	12.9			13.7	14.6			+
	28	3.0	3.9			2.8	2.9			—
	29	8.4	8.6			6.9	6.7			—
	30	7.1	5.8			5.2	4.3			—
	31	16.2	20.4			19.7	19.6			+
	32[a]	143	145			108	92			—
Arith. mean			26.86				21.26			—
Geom. mean			2.529				2.427			—
E	33[a]	104	110	106	115	120	111	123	98	+
	34[a]	210	242	270	265	274	302	232	237	+
	35	293	263	259	240	27.1	24.9	26.0	25.8	—
	36[a]	72	69	53	77	63	72	76	82	+
	37[a]	161	156	141	133	160	159	141	142	+
	38	8.3	11.0	9.2	10.3	9.9	9.5	10.5	8.3	—
	39	23.1	22.4	23.7	27.0	23.8	23.7	22.7	24.6	—
	40	8.4	7.5	9.4	6.7	9.8	8.1	8.3	9.7	+
	41	18.5	15.2	20.5	18.6	24.0	26.7	12.9	19.7	+
Arith. mean			99.41				102.28			+
Geom. mean			3.930				3.972			+
F	42	18.5	20.6	20.2	21.1	24.0	20.8	23.2	22.8	+
	43	28.7	26.4	25.2	28.5	29.1	24.2	24.7	24.9	—
	44[a]	77	71	71	60	56	79	79	66	—
	45[a]	57	61	57	54	51	60	64	61	+
	46	6.8	5.1	6.4	5.7	6.3	7.5	8.0	8.1	+
	47	6.7	6.6	6.4	6.9	6.5	7.2	9.4	7.0	+
	48	7.0	5.4	6.5	7.2	5.9	5.8	6.1	7.1	—
Arith. mean			27.64				28.38			+
Geom. mean			2.876				2.931			+
G	49	15.8	16.9	18.2	15.5	16.4	17.6	15.4	17.1	+
	50[a]	190	198	186	197	193	181	188	234	+
	51	14.0	15.1	18.0	18.7	16.3	16.6	17.1	17.2	+
	52	10.1	9.5	8.4	9.0	10.3	9.5	11.2	11.6	+
	53	9.5	11.2	10.5	10.0	9.0	9.5	7.8	8.6	—
	54	9.1	9.5	8.4	7.6	11.7	6.2	9.0	11.3	+
	55	3.4	3.2	2.3	2.7	3.2	3.2	3.6	3.3	+

TABLE 1. *Continued.*

Investigator	Milk sample no.	Phosphate (PB)				No phosphate (NPB)				Increase (+) or decrease (−) without phosphate[c]
		Rep. 1		Rep. 2		Rep. 1		Rep. 2		
	56	6.3	5.3	6.4	4.7	6.2	5.7	5.0	5.1	−
	57	2.7	3.6	1.7	2.6	2.3	2.1	3.3	3.3	+
	58[a]	44	32	41	39	34	39	47	41	+
	59[a]	123	141	132	149	131	158	120	149	+
	60[a]	200	239	239	218	222	251	259	231	+
	61[a]	170	188	138	172	140	164	162	176	−
	62[a]	234	194	203	241	202	247	249	230	+
	63[a]	102	121	121	118	124	110	120	113	+
	64[a]	268	288	279	251	282	288	281	269	+
	65[a]	135	140	157	176	153	180	130	141	−
	66[a]	249	226	231	261	279	242	261	258	+
	67[a]	230	238	241	221	245	223	209	221	−
Arith. mean			108.56				111.48			+
Geom. mean			3.792				3.818			+
H	68	6.0	6.4			7.0	7.9			+
	69[a]	59	65			74	69			+
	70[a]	168	160			175	163			+
	71	5.4	6.2			5.6	6.4			+
	72	8.4	8.2			6.1	7.0			−
	73	10.6	9.4			8.9	10.0			−
	74	8.5	8.2			9.8	9.8			+
	75	6.6	7.1			7.9	6.8			+
	76	7.4	6.6			6.8	6.2			−
Arith. mean			30.94				32.62			+
Geom. mean			2.579				2.606			+
I	77	5.0	5.9			6.3	5.9			+
	78	7.0	6.4			6.8	6.7			+
	79	10.2	9.5			10.0	9.1			−
	80	9.3	8.7			10.0	10.1			+
	81	6.4	6.3			6.8	6.5			+
	82	7.0	6.5			6.4	5.9			−
Arith. mean			7.35				7.54			+
Geom. mean			1.972				1.998			+
All samples										
Arith. mean			54.00				54.64			+
Geom. mean			3.037				3.046			+

[a]These milk samples were "high count" with the 10^{-3} dilution used for enumeration.
[b]For actual counts per ml milk, multiply by 1000.
[c]Based on geometric means.
[d]Natural log.

7.10, 7.20, 7.35, and 7.5 for four other samples. The two highest were those having the highest initial pH values. Addition of 1 ml of milk to the unbuffered water brought the pH values to a range of 6.8 to 7.2. Addition of milk to the buffered dilution blanks resulted in a very slight drop in pH (about 0.1 unit) in most instances.

Analysis of paired differences

The original plate count values are shown in Table 1. The overall mean counts of NPB were very slightly greater than PB. Eight and seven of the nine analysts reported higher mean counts with NPB dilutions for arithmetic and geometric means, respectively. Of the 82 milk samples tested, the geometric means of plate counts in NPB dilution blanks were greater than PB in 50 cases. Using a nonparametric sign test for matched pairs (5), the differences between NPB as opposed to PB, as shown in Table 1, were found to be significant ($0.05 < P < 0.01$). A t-test of paired differences of the proportion $\frac{\text{NPB-PB}}{\text{PB}}$, transformed to $y = 2 \arcsin \sqrt{x}$ (for normalizing the distribution), also showed the two diluents to give significantly different means ($P < 0.05$).

Since a significant amount of milk phosphate could be carried over in the first dilution and less in the second, the possibility existed that high count milk, i.e. that with counts over 30,000/ml, would behave differently from lower count milk. Analyses of these milk samples showed NPB > PB in 20/30 high count and 30/52 low count samples. A Chi square test of goodness-of-fit, however, did not indicate these ratio differences to be significant.

Analysis of variance

An examination of the differences between plate counts of NPB and PB in Table 1 shows that analyst B found 7 of 8 samples with PB > NPB; although the overall arithmetic mean showed a reverse trend due to the 8th sample. Analyst C found all 6 samples with NPB > PB. The large discrepancy between these two analysts (the

TABLE 2. *Analysis of variance determinations for 82 milk samples*

Line	Source of variation	df	Sum of squares	Mean square	F	Significant with $P<0.05$	$P<0.01$
A	Analysts	8	366.226	45.7782	3.44	yes	yes
B	Samples/analysts	73	972.063	13.3159	914	yes	yes
C	Treatment (phosphate versus no phosphate)	1	0.020427	0.020427	1.40	no	no
D	Treatment × analysts	8	1.02921	0.128651	8.84	yes	yes
E	Treatment × samples/analysts	73	1.39636	0.019128	1.31	no	no
F	Between replicates/samples	164	2.38750	0.014558	1.63	yes	yes
G	Between petri dishes	328	2.93500	0.008948			
	Total	655	1346.06				

F-values were derived from the ratios of the mean squares of lines A/B, B/F, C/F, D/F, E/F, F/G.

TABLE 3. *Analysis of variance of variances between duplicate petri dishes for 82 milk samples*

Line	Source of variation	df	Sum of squares	Mean square	F-ratio	Significant with $P<0.05$	$P<0.01$
A	Analysts	8	6171.82	771.478	1.58	no	no
B	Samples/analysts	73	36144.2	495.126	1.40	yes	no
C	Treatment	1	50.5766	50.5766	0.54	no	no
D	Treatment × analysts	8	730.255	91.2819	0.22	no	no
E	Treatment × samples/analysts	73	31071.4	425.636	1.20	no	no
F	Between replicates/samples	166	58894.8	354.788			
	Total	327	133063.1418				

F-values were derived from the ratios of the mean squares of lines A/B, B/F, C/F, D/F, E/F.

other seven analysts showed a more random distribution of differences) indicated either chance differences or some personal, geographical, or other bias. The analysis of variance is designed to determine the true nature of such differences. The analysis of data of Table 1, transformed to natural logs, is shown in Table 2. There was, as expected, a highly significant difference between milk samples (line B). There was also a highly significant ($P < 0.01$) difference between analysts (line A) which might have been due to the particular samples analyzed. There was no significant difference between NPB and $\overline{\text{PB}}$ (line C). There was a highly significant interaction ($P < 0.01$) between treatments and analysts (line D). This probably accounts for the apparent significant difference obtained between NPB and PB with the nonparametric and t-tests when applied to the data of Table 1 (these tests do not detect interaction effects). There was no evidence that the higher means obtained using NPB were different from PB. The test for interaction between phosphate treatment and samples within individual analysts (line E) was not significant at the 5% level. Replicates of the same milk sample (line F) were significantly more variable than the variability between petri dishes of the same replicate (line G).

Test for reproducibility

A single degree-of-freedom variance between petri dishes was calculated for each replication of each sample. These differences were examined by analysis of variance. The results are summarized in Table 3. The only difference in reproducibility was in samples within analysts (line B) which showed a difference with $P < 0.05$ but not with $P < 0.01$. In other words variances between milk samples were different and were not associated with any particular analyst. It was interesting to note that in this study there was no difference in analyst reproducibility, although we had previously (*6*) found one analyst to have a significantly better reproducibility. This analyst did not participate in this current study.

DISCUSSION

The results we obtained, as reported here, do not indicate any advantage for using phosphate buffer in the dilution blanks. There was some evidence from the less powerful statistical methods used that the counts were higher without phosphate. It is possible, though, that where further dilutions are necessary, some advantage might accrue from the use of phosphate. Under most conditions, only one dilution of 10^{-2} is made for raw milk. Our results are based on this dilution.

Wagenaar and Jezeski (*10*) studied the survival of *Pseudomonas putrefaciens* in distilled water and in water buffered with gelatin phosphate (0.2% gelatin, 0.725% $NaH_2PO_4 \cdot H_2O$) and 0.37% Na_2HPO_4). They reported differences in survival of several strains in distilled water and in the case of the most resistant strain, found that gelatin phosphate greatly improved survival with phosphate being the active component. the best phosphate concentration was 2% with progressively less survival at 1 and 0.5%. *Standard Methods,* however, recommends a phosphate concentration of only about 0.04%. Atherton (*2*) found some evidence that increasing dilutions of 12-day stored pasteurized milk resulted in lack of growth of psychrotrophic bacteria presumably because of a need for phosphate (carried over by the milk at low dilutions) as a growth stimulant. He indicated, however, that in general the psychrotrophic bacteria were not noticeably affected by the lack of phosphate in the dilution water. Watrous (private communication, 1973) indicated that the bacterial counts on stored processed dairy products such as pasteurized milk might be influenced by the presence or absence of phosphate. Other workers produced evidence that demineralized or distilled water decreases survival of pure cultures of *Escherichia coli* (*4*) and *Streptococcus faecalis* R (*8*).

It is apparent from our studies that the present use of phosphate in raw milk dilution blanks for the standard plate count is of no value in increasing the counts of bacteria or increasing reproducibility. It is possible that a dilution fluid similar in composition to the minerals of milk would be of more value for standard plate counts of the bacteria in dairy products. Peptone water should also be evaluated as a possible diluent for raw milk bacteria. Studies of such diluents are contemplated by our subcommittee.

REFERENCES

1. American Public Health Association. 1972. Standard methods for the examination of dairy products, 13th ed. American Public Health Association, Inc., New York, New York.
2. Atherton, H. V. 1953. Some physical, chemical, and bacteriological changes in market milk during prolonged periods of refrigerated storage. Thesis, The Pennsylvania State College, State College, Pa.
3. Butterfield, C. T. 1932. The selection of a dilution water for bacteriological examinations. J. Bacteriol. 23:355-368.
4. Carlucci, A. F., and D. Pramer. 1960. An evaluation of factors affecting the survival of *Escherichia coli* in sea water. II. Salinity, pH, and nutrients. Appl. Microbiol. 8:247-250.
5. Hays, W. L. 1963. Statistics. Holt, Rinehart and Winston, Inc., New York, New York.
6. Huhtanen, C. N., A. R. Brazis, W. L. Arledge, E. W. Cook, C. B. Donnelly, R. E. Ginn, J. N. Murphy, H. E. Randolph, E. L. Sing, and D. I. Thompson. 1970. Effect of dilution bottle mixing methods on plate counts of raw-milk bacteria. J. Milk Food Technol. 33:269-273.
7. Mohlman, F. W., G. P. Edwards, and G. Swope. 1928. Technic and significance of the biochemical oxygen demand determination. Ind. Eng. Chem. 20:242-246.
8. Price, S. A., and L. Gare. 1959. A source of error in microbiological assays attributable to a bacterial inhibitor in distilled water. Nature 183:838;840.
9. Theriault, E. J., P. D. McNamee, and C. T. Butterfield. 1931. Experimental studies of natural purification in polluted water. V. Selection of dilution waters for use in oxygen-demand tests. Pub. Health Report 46:1084-1116.
10. Wagenaar, R. O., and J. J. Jezeski. 1952. The influence of the type of diluent on the growth and survival of *Pseudomonas putrefaciens*. J. Dairy Sci. 35:738-754.